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The imprinted *Air* ncRNA is an atypical RNAPII transcript that evades splicing and escapes nuclear export

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Expression of the *Air* ncRNA is necessary to silence multiple genes in *cis* in the imprinted *Igf2r* cluster. However, its mode of action is unknown. Here, we characterize co- and post-transcriptional features of *Air* that identify it as a new member of the class of nuclear regulatory RNAs. We show that *Air* is transcribed from a DNA methylation-sensitive promoter by RNA polymerase II (RNAPII). However, although it is capped and polyadenylated similar to other RNAPII transcripts, the majority of *Air* transcripts evade cotranscriptional splicing resulting in a mature 108 kb ncRNA. As a consequence, the mature unspliced *Air* is nuclear localized and highly unstable. These features show that *Air* is an atypical RNAPII transcript whose properties indicate that its mode of action in gene silencing may not depend on the RNA *per se* but instead is related to its actual transcription.

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Introduction

A surprisingly large number of noncoding RNAs (ncRNAs) that have been suggested to constitute a new epigenetic regulatory system have recently been identified by diverse transcriptome analyses in mammalian cells (Mendes Soares and Valcarcel, 2006). Functional classification of these ncRNAs has not yet started but categories with known gene regulatory functions include short or micro-ncRNAs that participate in RNA interference and post-transcriptional regulatory pathways, intergenic transcripts that regulate local chromatin activity, *cis*-acting long ncRNAs such as *Xist* involved in chromosome inactivation and the imprinted *Air* and *Kcnq1ot1* ncRNAs involved in domain silencing (Heard, 2004; Pauler and Barlow, 2006).

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The *Air* ncRNA regulates genomic imprinting of a cluster of autosomal genes on mouse chromosome 17 (Braidotti *et al*, 2004). Many details of the epigenetic mechanism regulating genomic imprinting in mammals have been described, and it is now appreciated that most imprinted genes are found in clusters that contain at least one long ncRNA (Verona *et al*, 2003). In a typical mammalian imprinted gene cluster of which there are six well-characterized examples, the expression of multiple imprinted protein-coding genes is restricted to one parental chromosome, whereas the other parental chromosome silences these mRNA genes and instead expresses an ncRNA. Thus, imprinted ncRNAs show discordant expression *in cis* with imprinted protein-coding mRNA genes, indicating that they may act as silencers. Of the three imprinted ncRNAs so far tested, a silencing function has been shown for the *Air* and *Kcnq1ot1* ncRNAs (Sleutels *et al*, 2002; Mancini-Dinardo *et al*, 2006). The *H19* ncRNA, however, does not act as a silencer, despite showing a similar discordant expression pattern (Schmidt *et al*, 1999). Although a direct role for other imprinted ncRNAs has not yet been tested, deletion of the ncRNA promoters in three other imprinted clusters is associated with loss of silencing of *cis*-linked imprinted mRNA genes, indicating that these ncRNAs may also possess silencing functions (Chamberlain and Brannan, 2001; Lin *et al*, 2003; Williamson *et al*, 2006).

Targeted manipulations in mice have shown that expression of the 108 kb long *Air* ncRNA exerts a silencing effect *in cis* on three protein-coding genes *Igf2r*, *Slc22a2* and *Slc22a3*, spread over 300 kb (Sleutels *et al*, 2002). Thus, *Air* exerts a domain silencing effect (Figure 1A). It is not yet known if *Air* is needed to induce silencing, or acts at a later stage to maintain the silenced state. It is also unknown if silencing requires the *Air* RNA itself or, merely, the act of *Air* transcription through its 108 kb locus. The *Air* promoter is located in the second intron of *Igf2r* and transcription occurs only on the paternal chromosome in antisense orientation through the *Igf2r* promoter, terminating 108 kb downstream in the last intron of the next flanking gene, *Mas1* (Figure 1A and B).

The mature *Air* transcript is polyadenylated but, in contrast to most mature transcripts, is rich in interspersed repeats and appears to have few or no introns (Lyle *et al*, 2000). The other two genes silenced by *Air* (*Slc22a2* and *Slc22a3*) do not overlap nor share sequence homology of single-copy DNA with *Air*. Silencing by *Air* is also regulated in a tissue- and developmental-specific manner in the brain and placenta, the basis of which is unknown (Sleutels *et al*, 2002; Yamasaki *et al*, 2005). The *Air* promoter is silent on the maternal chromosome that expresses the three protein-coding genes and carries a DNA methylation imprint that is established during oocyte development (Stoger *et al*, 1993). In agreement with the finding that expression of *Air* silences genes on the paternal chromosome, we can reason that DNA

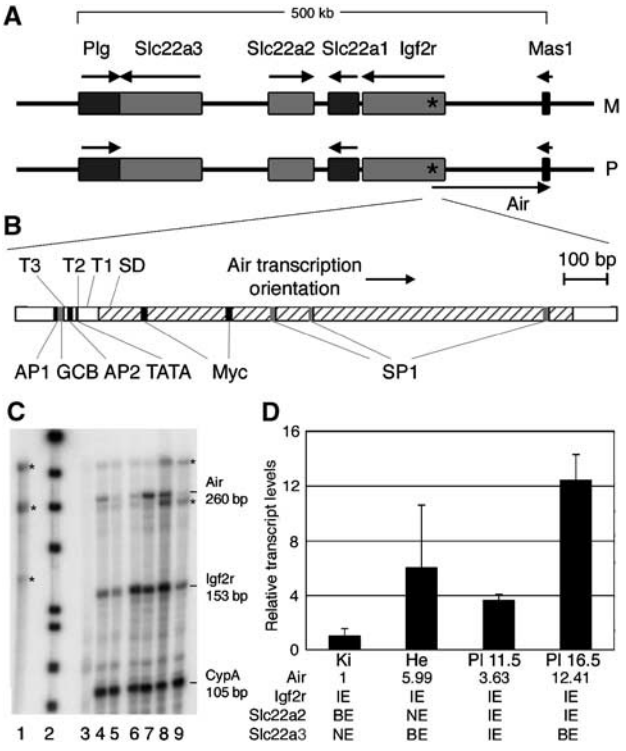


Figure 1 The *Air* ncRNA gene and promoter. (**A**) Map showing the position and orientation of *Air* ncRNA relative to flanking genes. The *Air* promoter lies in *Igf2r* intron 2 and the 3′-end in the last *Mas1* intron. Arrows: expressed genes; P: paternal allele; M: maternal allele; *: *Air* promoter. (**B**) Promoter organization of *Air*. Stripes indicate the *Air* CpG island (http://www.ebi.ac.uk/emboss/cpgplot/), T1–3: transcription starts; SD: splice donor site described in Figure 5. Consensus sites are indicated for AP1, AP2, SP1, Myc, GC-box (GCB) and a TATA sequence. (**C**) Steady-state levels of *Igf2r* and *Air* determined by RPA in cells and adult mouse using probe F3B (*Air*), EX46/47 (*Igf2r*): 1: probe + yeast RNA−RNase; 2: size marker; 3: probe + yeast RNA + RNase; 4: adult brain; 5: adult liver; 6: adult kidney; 7: adult lung; 8: adult heart; 9: NIH3T3. *CypA* (loading control). *Undigested probe. (**D**) QPCR (with q-assay *Air* middle) showing different *Air* transcript levels in adult mouse kidney (Ki), adult mouse heart (He), mouse placenta (Pl) 11.5 dpc and 16.5 dpc. *Air* transcript levels from four biological replicates were normalized to *18S* rRNA and levels in kidney were set to 1; the numbers below the bars indicate transcript levels relative to kidney. IE: imprinted expression; BE: biallelic expression; NE: not expressed.

methylation silences the *Air* promoter on the maternal chromosome to allow expression of the protein-coding genes. In support of this, it has been shown that mouse embryos lacking DNA methylation repress *Igf2r* on both parental chromosomes (Li *et al*, 1993). However, the effect of DNA methylation on *Air* expression has not yet been directly tested.

Although it is not yet known how *Air* expression leads to gene silencing, one model that is based on similarities between genomic imprinting and X-chromosome inactivation (XCI) in mammals is receiving much attention. These similarities arise from the observation that both imprinting and XCI use an epigenetic *cis*-acting silencing mechanism dependent on an ncRNA (named *Xist* in XCI), that XCI is also an imprinted phenomenon in marsupials and in extra-embryonic mouse tissues, and that both imprinting and XCI share a similar evolutionary distribution (Reik and Lewis, 2005). If the *Air* ncRNA performed a function similar to that of the *Xist* transcript in XCI, it would be expected to coat the 300 kb region containing the silenced genes and to recruit repressive chromatin as has been described for the action of *Xist* (Heard, 2004). The small size of the genomic region silenced by the *Air* ncRNA precludes much of the immunofluorescent analyses that have been performed for the *Xist* RNA to show recruitment of repressive chromatin to the whole 180 Mbp X chromosome. Conversely, the large size of the *Air* RNA makes it laborious to define functional regions along its 108 kb length as has been performed for the 17.9 kb *Xist* RNA (Wutz *et al*, 2002).

A more detailed understanding of the transcriptional and post-transcriptional features of the *Air* ncRNA may, however, help to distinguish which model of *Air*-mediated gene silencing is operating. Here, we report that the *Air* promoter is transcribed by RNA polymerase II (RNAPII) in a DNA methylation-sensitive manner and, similar to other RNAPII transcripts, has a 7mGcap. However, in contrast to most RNAPII transcripts, we find that *Air* is inefficiently spliced such that most nascent transcripts constitute the unspliced mature 108 kb form of this ncRNA. The unspliced 108 kb *Air* ncRNA is highly unstable relative to the *Igf2r* mRNA and is not exported to the cytoplasm, whereas the spliced *Air* variants have a similar stability to the *Igf2r* mRNA and are exported. The data presented here show that *Air* is a new member of the nuclear regulatory RNA family, whose properties indicate that its ability to silence genes in *cis* may not depend on the RNA itself but, instead, may be related to transcription through its 108 kb locus.

Results

Expression and abundance of the Air ncRNA

The organization of the imprinted *Igf2r* cluster and details of the *Air* promoter are shown in Figure 1A and B. RNase protection assays (RPA) were used to determine the steady-state levels of *Igf2r* and *Air* in adult mouse tissues and cell lines expressing different levels of these two genes. A multiprobe RPA was performed that simultaneously detected *Air*, *Igf2r* and *cyclophilin A (CypA)* transcripts. *Air* could be detected in all tissues examined, but was consistently less abundant than *Igf2r* in all tested tissues (Figure 1C). The levels of *Air* relative to *Igf2r* varied between different tissues and cell types from 17% (adult kidney) to 67% (adult lung) (Figure 1C, lanes 6 and 7). Similar ratios were found using two different *Air* and *Igf2r* probes (data not shown). The reason for the differing ratios of *Igf2r*/*Air* in different cell types is not clear but may reflect single cell variation between cells that lack *Air* and express *Igf2r* biallelically, and cells that show imprinted expression of *Air* and *Igf2r*, as recently reported for mouse brain (Yamasaki *et al*, 2005). Figure 1D shows that the abundance of *Air* does not correlate with its silencing effects on genes within the *Igf2r* cluster. *Air* is equally abundant in the heart and 11.5 dpc placenta. However, only *Igf2r* shows imprinted expression in the heart, whereas *Igf2r*, *Slc22a2* and *Slc22a3* show imprinted expression in 11.5 dpc placenta (Sleutels *et al*, 2002). In 16.5 dpc placenta, only *Igf2r* and *Slc22a2* show imprinted expression but *Air* expression is three-fold higher compared to 11.5 dpc placenta.

The multiple transcriptional start sites (T1–3; Figure 1B) of the *Air* ncRNA are located at the 5′-border of a CpG island.

The CpG island is unmethylated on the paternal allele that expresses *Air* and methylated on the maternal chromosome that lacks *Air* expression. To test if DNA methylation represses *Air* on the maternal chromosome, we analysed embryos, which were wild-type, heterozygous or homozygous for a null mutation of *Dnmt1* (Li *et al*, 1993). Figure 2 shows the expression levels of four imprinted genes. The two protein-coding genes *Igf2r* and *Igf2* show reduced expression in *Dnmt1* null embryos compared to heterozygotes as previously described (Li *et al*, 1993). *Igf2* mRNA levels are reduced to background in this assay, whereas *Igf2r* mRNA levels are reduced to 26% of controls. In contrast, the two ncRNAs *Air* and *H19* show more than two-fold increased expression in *Dnmt1* null embryos compared to heterozygotes. The increase in *H19* ncRNA level has previously been shown to occur from re-expression of the normally silent paternal *H19* promoter (Li *et al*, 1993). These results show that the *Air* ncRNA promoter is repressed by DNA methylation in mouse embryos.

Air is a non-coding RNAPII transcript

To determine which RNAP transcribes *Air*, we made use of α-amanitin, which inhibits RNAPII at low (5 μg/ml) concentrations. RNAPIII can also be inhibited by high concentrations, whereas RNAPI remains unaffected by α-amanitin. Figure 3A shows that at 5 μg/ml α-amanitin, the RNAPII-transcribed *Myc* gene is fully repressed at 24 h, whereas the RNAPIII *5S* RNA promoter is not affected. The same RNA preparation analysed in Figure 3A was then used to quantify *Air* by qPCR, which shows that *Air* levels decrease to 13–30% by 24 h treatment (Figure 3B). This indicates that the *Air* ncRNA is transcribed by RNAPII.

We next investigated if the *Air* ncRNA is modified with a methyl-7-guanosine (7mGcap) characteristic of RNAPII transcripts. We used a monoclonal antibody to immunoprecipitate capped RNAs using the capped *Igf2r* mRNA as a positive control, and the uncapped ribosomal *18S* rRNA as a negative control to monitor unspecific binding of RNA to the antibody. Figure 4A shows that *Air* and *Igf2r* are found in the immunoprecipitated fraction, whereas the *18S* rRNA is not. The efficiency of immunoprecipitation (IP) of *Air* and *Igf2r* relative to *18S* rRNA shows a large variation for the three biological replicates (Figure 4B). However, control reactions lacking antibody or with an unrelated antibody of the same IgG isotype (Figure 4C and D) consistently yielded only a low level of unspecific bound RNA. Therefore, these results demonstrate that the *Air* ncRNA carries a 7mGcap.

Air has reduced splicing potential

Air was initially reported as an unspliced RNA (Lyle *et al*, 2000). To investigate whether any *Air* transcripts are spliced, EST databases were scanned for discontinuous alignments to the *Air* sequence. A total of 204 EST sequences were found and of these, 13% showed a discontinuous alignment indicating that they spanned an intron. A total of 13 independent clones were identified that showed the same orientation as the *Air* ncRNA and were classified into five groups

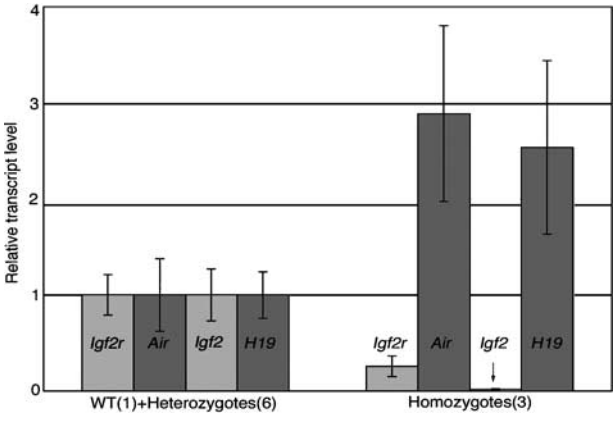


Figure 2 The *Air* ncRNA promoter is repressed by DNA methylation. Expression of *Igf2r* (q-assay ex48), *Air* (q-assay *Air* middle), *Igf2* (q-assay *Igf2*) and *H19* (q-assay *H19*) was assayed by qPCR in E9.5 embryo cDNAs from one litter containing one wild-type (WT), six heterozygotes and three homozygotes for a *Dnmt1* null mutation (Li *et al*, 1993). RNA from individual embryos was assayed separately and used to calculate the standard deviation. Values are normalized to *Gapdh*. Results from WT and heterozygous embryos were similar and were pooled and set to 1. *Igf2r* and *Igf2* mRNAs show a reduction in *Dnmt1* null embryos of 0.26 and 0.02 (arrow), respectively. *Air* and *H19* ncRNAs show enrichments of 2.89 and 2.54, respectively.

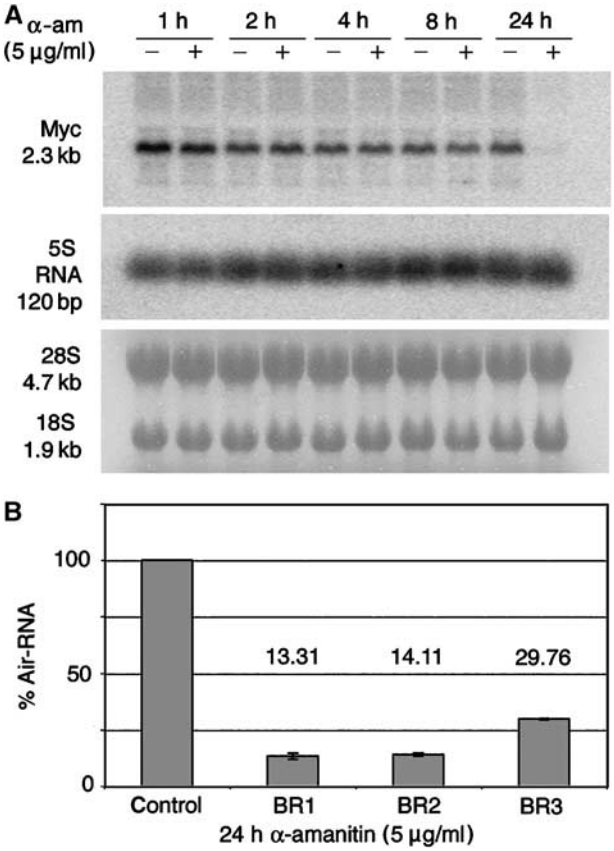


Figure 3 The *Air* ncRNA is an RNAPII transcript. (**A**) RNA blot of NIH3T3 cells exposed to α-amanitin (α-am). Total cell RNA from control (−) and poisoned (+) cells was hybridized with the RNAPII transcript *Myc* and the RNAPIII transcript *5S* RNA (probes are listed in Supplementary data). One of three biological replicates is shown demonstrating inhibition of RNAPII but not RNAPIII transcription at 24 h. Methylene blue staining of the 28S and *18S* rRNA bands is shown underneath as loading control, as RNAPI is not affected by α-am. (**B**) *Air* expression analysed by qPCR (q-assay *Air* middle) normalized to *18S* rRNA shows reduced *Air* expression in all three biological replicates (BR). The untreated control was set to 100%.

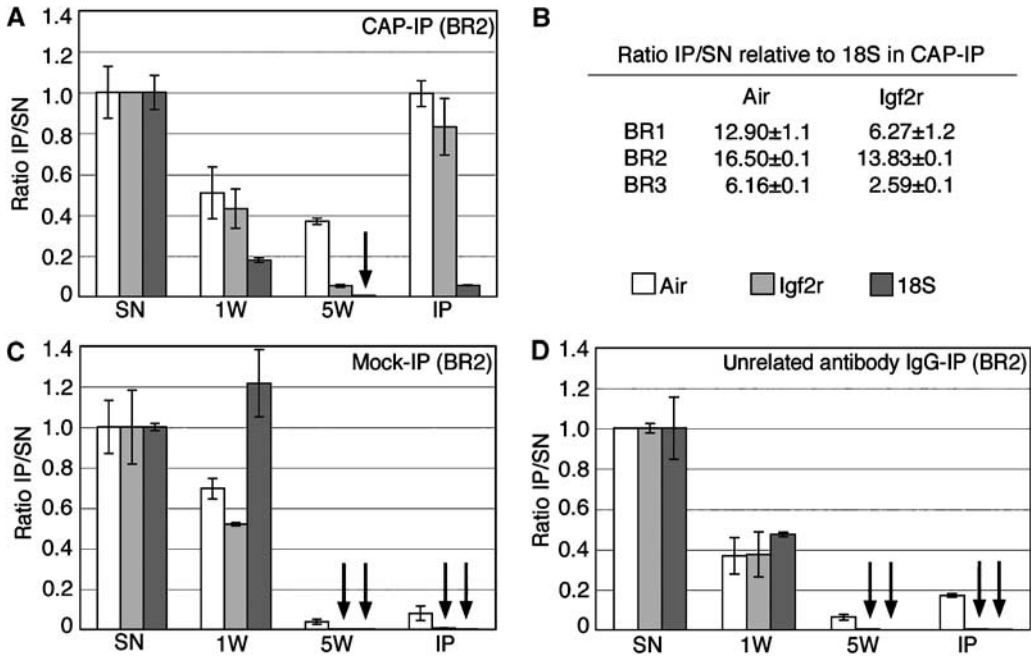


Figure 4 The *Air* ncRNA bears a 7mGcap. (**A**) qPCR of RNA immunoprecipitated (IP) by the cap-specific antibody H20. RNA from four fractions from biological replicate 2 (BR2), supernatant (SN), first wash (1W), fifth wash (5W) and antibody-bound RNA (IP), was analysed by qPCR for *Air* (white bars, q-assay *Air* middle), *Igf2r* (light grey bars, q-assay ex48) and *18S* (dark grey bars, q-assay 18S). The SN fraction was set to 1 and the other fractions are displayed as relative enrichment/reduction. Arrows indicate a negligible value. (**B**) Ratio of IP RNA to RNA in the supernatant (IP/SN) is shown for *Air* and *Igf2r* for three biological replicates relative to the same ratios for *18S* rRNA. (**C**) Control reactions for beads only without antibody (Mock-IP) used to monitor unspecific binding to Sepharose G-beads. (**D**) IP with an unrelated antibody of the same isotype (IgG-IP) used to determine unspecific binding of RNA to the IgG epitope.

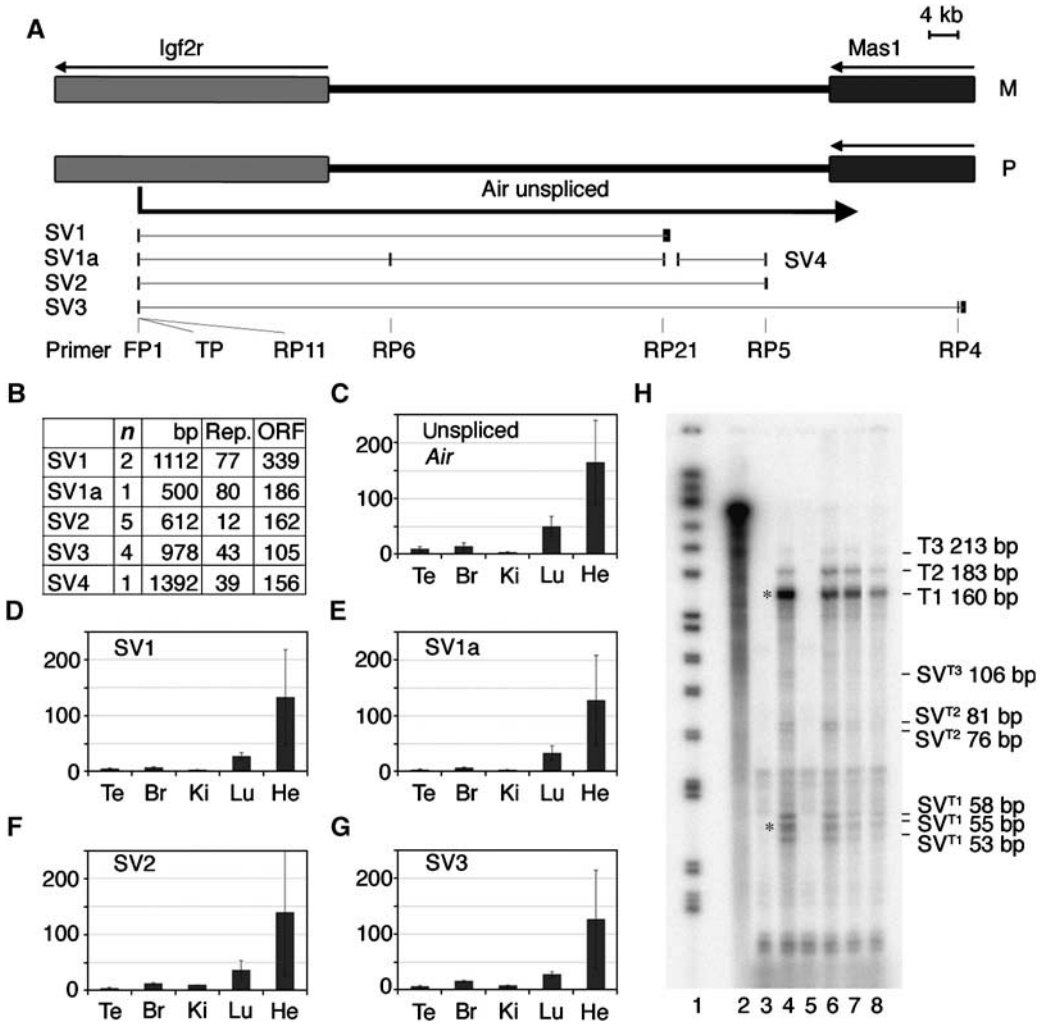
(splice variant (SV)1 (DQ275617), SV1a (DQ275618), SV2 (DQ275619), SV3 (DQ275620), SV4) whose organization and general features are shown in Figure 5A and B. All splice variants except SV4, whose 5′-end is missing, have their 5′-end close to the *Air* main transcription start (T1) and all use the same splice donor 53 bp downstream of T1 (SD; Figure 1B). Therefore, all splice variants share the first exon.

Splice variants SV1 and SV1a share a common downstream exon, whereas the remaining variants have different downstream exons. None of the splice variants end at the same polyadenylation site identified for full-length unspliced *Air*. The 3′-ends of SV1 and SV3 are A-rich and these clones may have been obtained by oligo-d(T) priming of internal stretches of multiple A's. However, SV2 and SV4 both end in a region without obvious enrichment of A's. It is notable that one splice variant (SV3) has its transcription end more than 17 kb downstream of the mapped 3′-end of the full-length unspliced *Air* (at 929 bp in sequence AJ249895). The existence of the previously described polyadenylation site (18 464 bp in AJ249895) was confirmed in mouse tissues and cells used here (data not shown) (Lyle *et al*, 2000). The lengths of the spliced variants range from 500 to 1392 bp and the longest open reading frames range from 105 to 339 bp; however, all contain interspersed repeats (12–77%) (Figure 5B). This indicates that these spliced variants are likely to be ncRNAs.

The relative expression levels of unspliced and spliced *Air* transcripts were determined in organs that show imprinted *Igf2r* expression (kidney, lung, heart) and in organs that partly lack imprinted expression (testis, brain). Figure 5C–G shows that similar relative amounts of unspliced and spliced *Air* are found in all tested tissues, indicating a lack of correlation between splicing of *Air* and loss of *Igf2r* imprinted expression. We used RPA to quantify the proportion of *Air* that is spliced (Figure 5H), using a probe that overlaps the three known transcriptional start sites and the common 53 bp splice donor. Both unspliced and spliced *Air* are only expressed from the paternal (Figure 5H, lanes 4 and 6) and not from the maternal chromosome (lane 5). As all splice variants use the same exon 1 splice donor, the RPA probe detects the sum of splice variants (bands labelled SV in Figure 5H). All three transcriptional start sites could be detected for unspliced (T1, T2, T3) and spliced *Air* ncRNA (SV^{T1}, SV^{T2}, SV^{T3}). However, the main transcriptional start site for the spliced variants (SV^{T1}) is seen as three separate bands and the second start site SV^{T2} is also seen as two faint bands on the original image, which indicates that these start sites are used with loose stringency. The abundance of steady-state spliced relative to unspliced *Air* was determined as between 23 and 44% (Figure 5H).

The Air ncRNA is not exported to the cytoplasm

Nuclear, cytoplasmic and total cellular RNAs were analysed to test cellular localization of *Air*. Figure 6 shows the result of two experiments using a qPCR assay and RNA blots. In the qPCR assays, all values are normalized to *CypA* and expressed as enrichment relative to this mRNA and for each histogram the value in the total cellular RNA is set to 1. Thus, an mRNA localized to the cytoplasm such as *Gapdh* shows no enrichment relative to *CypA*, and cytoplasmic and nuclear levels approximately equal 1 (Figure 6A). In contrast, the nuclear-localized *45S* rRNA shows a mean nuclear/cytoplasmic ratio of 621:1 (Figure 6A). The imprinted *H19* ncRNA shows a mean nuclear/cytoplasmic ratio of 1.0:1,



	n	bp	Rep.	ORF
SV1	2	1112	77	339
SV1a	1	500	80	186
SV2	5	612	12	162
SV3	4	978	43	105
SV4	1	1392	39	156

Figure 5 *Air* has reduced splicing potential. (**A**) Map showing relative positions *Air*, *Igf2r* and *Mas1*. Indicated below are full-length unspliced *Air* and five groups of spliced variants (SV1, SV1a, SV2, SV3, SV4) that share the same 5′-splice donor 53 bp downstream of the transcriptional start. Black bars: exons; grey lines: introns; locations of primers (one forward primer (FP) combined with different reverse primers (RP)) and Taqman probe (TP) used for qPCR are shown underneath. (**B**) Summary of spliced *Air* EST clones found in databases (www.ensembl.org and http://www.ncbi.nlm.nih.gov/BLAST). *n*: number of clones; bp: length of SV; Rep: % repeats determined by http://www.repeatmasker.org/; ORF: length of longest open reading frame. (**C–G**) qPCR assays showing relative expression levels (normalized to cyclophilin q-assay *CypA* ex3/4) in adult mouse tissues of unspliced (C, q-assay *Air* 5′) and spliced (D-G q-assays SV1, SV1a, SV2, SV3) *Air* ncRNA. Te: testis; Br: brain; Ki: kidney; Lu: lung; He: heart. (**H**) RNA prepared from cell lines and tissues was analysed by RPA with probe MlMs1 spanning the multiple *Air* transcription start sites showing spliced and unspliced *Air* transcripts. 1: size marker; 2: probe + yeast RNA − RNase; 3: probe + yeast RNA + RNase; 4: Thp/DB104 MEFs; 5: DB104/Thp MEFs; 6: MEFF Thp/+ cells; 7: NIH3T3 cells; 8: adult heart from wild-type mice. The maternal allele is written on the left side. See Materials and methods for details of cells. Transcription starts for unspliced *Air*: T1, T2 and T3; for spliced *Air*: SVT1, SVT2 and SVT3. Protected bands for SVT2 and SVT3 are visible on original exposures. Using bands marked by an asterisk in lane 4, the abundance of spliced *Air* is 23–44% that of unspliced *Air* in lanes 4, 6, 7 and 8.

confirming a previous report that it is exported to the cytoplasm (Brannan *et al*, 1990) (Figure 6A). Analysis of the unspliced *Air* ncRNA in these same samples shows a mean nuclear/cytoplasmic ratio of 55:1, indicating that unspliced *Air* is nuclear localized (Figure 6B). The nuclear localization of *Air* can also be visualized by RNA blots, although this type of analysis is not efficient owing to the degradation of the 108 kb RNA (Figure 6C).

Igf2r mRNA assayed in the same RNA samples shows a mean nuclear/cytoplasmic ratio of 2.2:1 (Figure 6B, note the relatively strong nuclear signal for *Igf2r* and *Myc* compared to *Gapdh* and *CypA*). A similar result was obtained with two independent qPCR assays, RNA blots and the use of two different methods to prepare the nuclear and cytoplasmic RNA fractions (data not shown, see Materials and methods). In contrast to *Air*, however, *Igf2r* mRNA is clearly exported to the cytoplasm (Figure 6B and C). All *Air* splice variants are also exported with a similar efficiency as *Igf2r* (Figure 6D). This indicates that spliced *Air* in contrast to the majority of unspliced *Air* can be exported to the cytoplasm.

Unspliced Air is an unstable transcript

To determine the stability of the *Air* ncRNA, inhibition of total cellular transcription by Actinomycin D was used. *Myc* and *Gapdh* were chosen as controls for short and long half-life mRNAs. Figure 7A–C shows an RNA blot analysis of mouse embryo fibroblast (MEF) cells treated for up to 8 h with Actinomycin D demonstrating that *Myc* mRNA is largely

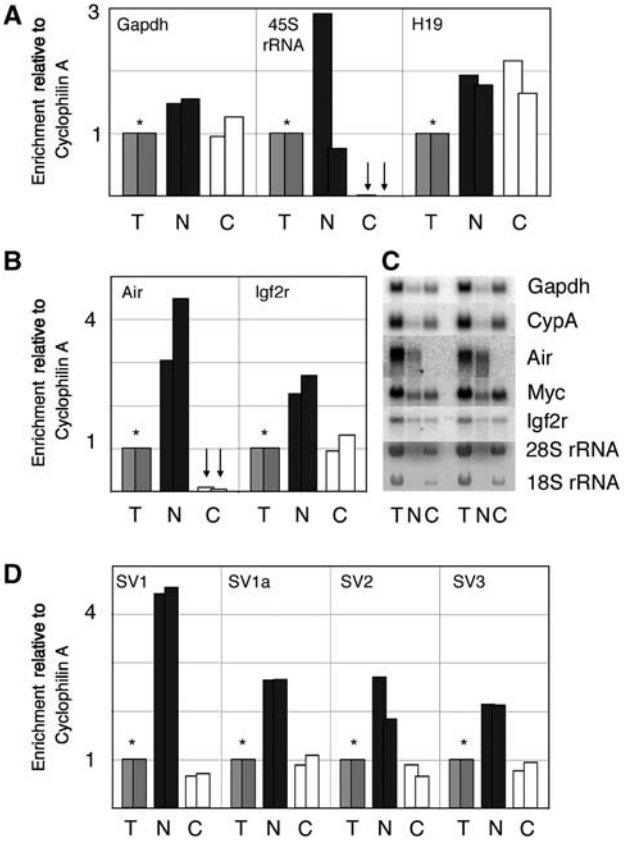


Figure 6 The unspliced *Air* ncRNA is not exported to the cytoplasm. qPCR assay of nuclear (N), cytoplasmic (C) and total cell (T) RNAs. Bars show values for two biological replicates normalized to *CypA*. The value in total RNA (T) is set to 1 (asterisk). Cyclophilin (*CypA*) is a known cytoplasmic mRNA. Other cytoplasmic mRNAs show the same distribution as *CypA* and show no enrichment in N and C fractions. (**A**) Distribution of control RNAs. *Gapdh* (q-assay *Gapdh* ex5) shows no enrichment in N and C relative to *CypA* (q-assay *CypA* ex3/4) and is located in the cytoplasm; *45S* pre-rRNA (q-assay *45S* pre-RNA) shows N/C ratios of 342/1 and 901/1 and is located in the nucleus; *H19* ncRNA (q-assay *H19*) shows N/C ratios of 0.9/1 and 1.1/1 and is located in the cytoplasm. (**B**) Distribution of unspliced *Air* (q-assay *Air* middle) shows N/C ratios of 30/1 and 80/1 and is located in the nucleus. *Igf2r* (q-assay ex48) shows N/C ratios of 2.4/1 and 2.1/1 and is exported to the cytoplasm but also present in the nucleus. (**C**) RNA blot of samples analysed in panel B showing the distribution of *Gapdh*, *CypA*, *Myc*, *Igf2r*, *Air* (probes listed in Supplementary data) and rRNA (methylene blue staining). (**D**) *Air* splice variants are exported to the cytoplasm and show similar N/C ratios as *Igf2r* (q-assay SV1: 6.8/1 and 6.4/1; q-assay SV1a: 3.0/1 and 2.4/1; q-assay SV2: 3.0/1 and 2.8/1; q-assay SV3: 2.8/1 and 2.2/1).

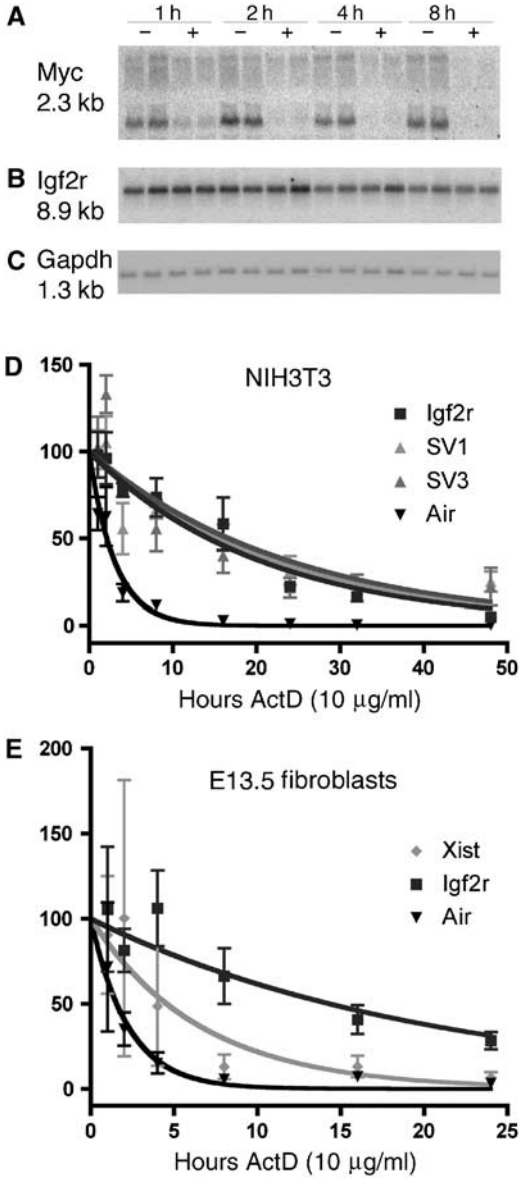


Figure 7 Full-length *Air* is an unstable transcript. (**A–C**) RNA blots of NIH3T3 exposed to 10 μg/ml of Actinomycin D (ActD). A representative set of blots from 1 of 3 biological replicates is shown. Total RNA was prepared from control (−) and poisoned (+) cells and hybridized to *Myc* (A), *Igf2r* (B) and *Gapdh* (C). Probes are listed in Supplementary data. *Myc* mRNA is depleted after 2 h, whereas *Igf2r* and *Gapdh* mRNA levels were unchanged after 8 h of ActD treatment. (**D**) qPCR assays for stability of the *Air* (q-assay *Air* middle) and *Igf2r* (q-assay ex48) transcripts using the RNA samples analysed in panels A–C. Each value was normalized to *Gapdh* (q-assay *Gapdh* ex5) (up to 8 h) or *18S* rRNA (q-assay 18S) (up to 48 h). Control untreated samples were set to 100% and ActD samples are shown as a % of controls. Values average three biological replicates each performed in technical duplicate. A one-phase exponential decay curve was calculated for these results and the half-life values are calculated by Prism4 (span = 100, plateau = 0, $k \geqslant 0$) as unspliced *Air* 2.1 h (black triangle), *Igf2r* 14.3 h (black square), SV1 15.4 h (light grey triangle) and SV3 16.7 h (dark grey triangle). (**E**) qPCR assays for stability of *Air*, *Igf2r* and *Xist* (q-assay Xist) with RNA of primary E13.5 dpc cells. Analysis was performed as in panel D; half-lives: *Air* 1.6 h (black triangle), *Igf2r* 14.3 h (black square), *Xist* 4.6 h (light grey square).

depleted after 1 h, whereas *Gapdh* and *Igf2r* show no significant changes. Figure 7D shows that the half-life of the unspliced *Air* ncRNA is 2.1 h, whereas that of *Igf2r* is 14.3 h. In contrast, the *Air* splice variants SV1 and SV3 show increased stability with half-lives of 15.4 and 16.7 h.

The above experiments were performed in established MEF cells between passages 12 and 20 that show full repression of the paternal *Igf2r* allele as determined by RNA FISH and acquisition of DNA methylation (Braidotti *et al*, 2004; data not shown). In order to investigate if *Air* shows a different stability at an earlier developmental time point when *Igf2r* silencing is incomplete in terms of repression and methylation (Stoger *et al*, 1993; Lerchner and Barlow, 1997), we investigated *Air* stability in cells obtained directly from 13.5 dpc embryos (Figure 7E). The half-lives for *Air* and *Igf2r* in these cells were similar (1.6 and 14.3 h, respectively) to those determined in established MEF cells. In order to

compare the stability of the unspliced *Air* ncRNA with that of a spliced ncRNA, we also determined the stability of the *Xist* ncRNA in these primary cells as 4.6 h (in agreement with a recent report; Sun *et al*, 2006), which is three-fold more stable than *Air* (Figure 7E).

Discussion

The Air ncRNA is an atypical RNAPII transcript

We show here that the *Air* ncRNA is transcribed by RNAPII and bears a 7mGcap and poly(A) tail, typical for such transcripts. However, it is atypical because although it shows some characteristics of RNAPII transcripts, it lacks others. *Air* transcripts mostly evade the splicing machinery, are unstable and are not exported to the cytoplasm. While there are some known unspliced mRNAs that are exported to the cytoplasm (Sakharkar *et al*, 2005), and two known spliced ncRNAs that are nuclear-localized (namely *Xist* and the *CTN*-RNA; Heard, 2004; Prasanth *et al*, 2005), the *Air* ncRNA is different as nuclear localization is only shown by unspliced variants, whereas the spliced variants are exported to the cytoplasm.

In addition to the three well-known mammalian RNAPs (RNAPI that transcribes ribosomal genes, RNAPII that transcribes mRNAs and ncRNAs, and RNAPIII that transcribes short tRNAs and small RNAs), a new nuclear-localized RNAP RNAPIV, which is an alternatively spliced product from the mitochondrial encoded RNAP gene, was recently found to be involved in the transcription of several mouse nuclear-encoded genes (Kravchenko *et al*, 2005). However, we can exclude that *Air* is transcribed by this polymerase, as RNAPIV was shown to be insensitive to α-amanitin. The finding that *Air* is transcribed by RNAPII is in agreement with bioinformatics analysis of the *Air* promoter that identified binding sites for typical RNAPII transcription factors such as SP1, commonly found at CpG islands (Figure 1B). It thus seems likely that long imprinted ncRNAs are generally transcribed by RNAPII, as the *Xist*, *Tsix* and *H19* ncRNAs are also products of RNAPII (Brannan *et al*, 1990; Navarro *et al*, 2005). The *H19* ncRNA was also shown to be modified with both 7mGcap and poly(A) tail (Pachnis *et al*, 1988). Both these 5′- and 3′-modifications are typical features of RNAPII transcripts (Vasudevan and Peltz, 2003).

Repression of an RNAPII promoter by DNA methylation

Silencing of a CpG island promoter by DNA methylation is an unusual mechanism in normal embryonic development and has only been found at imprinted and X-chromosome inactive genes (Antequera, 2003). In this study, we show that DNA methylation is necessary to silence *Air* expression in the mouse embryo. Although the *Igf2r* promoter becomes methylated in later embryonic stages, it has been previously shown that lack of DNA methylation reduces *Igf2r* transcription to very low levels (Li *et al*, 1993). This indicated, contrary to expectation, that DNA methylation was required for *Igf2r* expression. The observation in this study of a doubling of *Air* expression upon genomic demethylation, in combination with the previous demonstration that *Air* expression silences *Igf2r* in *cis* (Sleutels *et al*, 2002), explains this paradox.

Lack of nuclear export of an RNAPII unspliced transcript

The 108 kb unspliced *Air* is localized to the nucleus and not exported to the cytoplasm, in contrast to spliced *Air* variants and mRNAs such as *Igf2r* and *Myc*. It is generally considered that mRNAs are bound by multiple RNA-binding proteins during transcription, which are recognized by nuclear export factors and rapidly transported to the cytoplasm (Vasudevan and Peltz, 2003). However, mRNAs can show differing efficiencies in terms of nuclear export. As a result, mature transcripts for inefficiently exported mRNAs such as *C-jun* or β-*actin* can be found in almost equal amounts in both nuclear and cytoplasmic compartments, whereas efficiently exported mRNAs such as the *CypA* or *H4-histone* mRNAs are mostly found in the cytoplasm (Gondran *et al*, 1999). Our result shows that *Igf2r* mRNA and *Air* splice variants are exported, albeit inefficiently compared to *CypA*. However, unspliced *Air* completely escapes nuclear export.

The reason for nuclear retention of some mature mRNA transcripts is unclear. The lack of nuclear export of the full-length unspliced *Air* ncRNA can be explained neither by the fact that it is an ncRNA nor by its 108 kb size, as both the 2.4 kb *H19* and the ∼1000 kb *Ube3aAS* ncRNAs are efficiently exported to the cytoplasm (Brannan *et al*, 1990; Le Meur *et al*, 2005). Additionally, low stability cannot explain why unspliced *Air* is not detected in the cytoplasm, as mRNAs like Myc with five-fold lower stability are exported and detected in the cytoplasm (Gondran *et al*, 1999). It is important to note that single exon genes can also be efficiently exported to the cytoplasm (Sakharkar *et al*, 2005). Lack of splicing may, however, be a significant factor in the nuclear retention of the 108 kb full-length *Air*, as all four spliced variants are exported to the cytoplasm with efficiency similar to that of *Igf2r* and *Myc* mRNAs. This identifies splicing as a key regulator of *Air* ncRNA metabolism.

Inefficient splicing of an RNAPII transcript

It was previously shown that the 108 kb *Air* transcript was either completely unspliced or contained few and short introns (Lyle *et al*, 2000). Here we show that between 23 and 44% of *Air* steady-state level transcripts are spliced. The splice variants are, however, eight-fold more stable than full-length *Air*, which indicates that the amount of nascent transcripts that become spliced is a small minority (less than 5%) of total *Air* transcription. The majority of *Air* transcripts thus appear unspliced (with the caveat that small introns inside repetitive elements would not be detected in the EST screen).

The percentage of spliced *Air* in steady-state populations is similar to that found for the *Tsix* ncRNA that overlaps the *Xist* ncRNA in antisense orientation, where 30–60% of the transcripts are spliced and the function of the spliced product is unknown (Shibata and Lee, 2003). Many factors argue against a silencing role for the *Air* spliced variants. First, the sequence apart from the first 53 bp is different for each of the four spliced *Air* variants. Second, nuclear export of the spliced *Air* variants indicates that they would be unable to specifically target the paternal chromosome. Third, there is no correlation between expression of spliced variants and tissues that lack imprinted *Igf2r* expression. Last, it is unlikely that the splice variants are post-transcriptional regulators of cytoplasmic *Igf2r* mRNA, as they share no sequence homology with the mature transcript and a complete absence of

Air expression does not increase maternal *Igf2r* expression (Sleutels *et al*, 2002). All of these features argue against a functional role for the *Air* spliced variants.

It is unknown what prevents *Air* transcripts from being spliced—especially as multiple splice sites would be predicted to occur within 108 kb. We have previously shown that short *Air* mouse transgene constructs are spliced efficiently and that a rabbit β-*globin* splice acceptor inserted 3 kb downstream of the *Air* transcription start is used for 80% of transcripts (Sleutels and Barlow, 2001; Sleutels *et al*, 2002). We speculate that some sequence elements within the *Air* transcript, but outside the region contained in the above transgenes, could regulate the splicing ability of *Air*. The possibility that the atypical splicing behaviour of *Air* results from transcription in a nuclear compartment deficient in splicing factors also cannot be excluded, but appears less likely as in adult tissues the flanking non-imprinted *Slc22a1* gene is expressed and spliced from the paternal chromosome that also expresses the unspliced *Air* ncRNA.

The Air ncRNA is an unstable transcript

We show here that the unspliced *Air* ncRNA half-life is between 1.6 and 2.1 h and that it is approximately 10-fold less stable than the *Igf2r* mRNA that it silences. *Air* is also three-fold less stable in MEFs than the nuclear-localized *Xist* ncRNA. The ratio of stability between *Igf2r* and *Air* is reminiscent of what was recently described for the *Xist*/*Tsix* sense/antisense ncRNAs. The half-life of *Xist* was determined to be 3–4 h in fibroblast cells, whereas the half-life of unspliced *Tsix* was less than 1 h (the half-life of spliced *Tsix* has not been determined) (Sun *et al*, 2006). We show here that splicing of *Air* restores stability to a level comparable with that of the *Igf2r* mRNA. This may indicate that the 108 kb *Air* ncRNA is rapidly degraded because it is not spliced.

The co- and post-transcriptional features of the *Air* ncRNA described here show that *Air* is an inefficiently processed and unstable, nuclear-localized ncRNA. The possibility exists that these three features depend on each other. Absence of splicing could result in nuclear retention, as it has been shown that exon splice junctions are bound by exon junction complexes that enhance nuclear export (Vasudevan and Peltz, 2003). Absence of splicing may be a mechanism to trap the *Air* ncRNA close to the site of transcription to keep the silencing effect on the paternal chromosome and prevent it acting *in trans* on the maternal chromosome in the same nucleus. In support of this, it has been shown that β-*globin* mutants defective in splicing stay close to the site of transcription and are not exported to the cytoplasm (Custodio *et al*, 1999). The results presented here show that instability of the *Air* ncRNA is associated with a lack of splicing. Spliced *Air* variants are 10 times more stable but they are exported to the cytoplasm, away from the site of repression. This may indicate that *Air* transcription is more important than the ncRNA for its silencing function.

Models of Air-mediated gene silencing

Although it is not yet known how *Air* expression leads to gene silencing, three models have been considered (Sleutels *et al*, 2003). The first model proposes a form of 'expression competition' between *Air* and the three silenced protein-coding genes, which is based on common transacting factors required for promoter or enhancer activation (Barlow, 1997). This model is not supported by the demonstration that *Air* can be expressed *in cis* with the three protein-coding genes when truncated from 108 to 3 kb (Sleutels *et al*, 2002). The second model is based on the antisense orientation of *Air* with respect to *Igf2r* that results in a 28 kb transcription overlap between the two genes. In this 'sense–antisense' model, double-stranded RNA from *Igf2r*/*Air* transcription overlap could lead to RNAi–mediated silencing of the *Igf2r* promoter. The silencing of the *Igf2r* promoter would then lead in a second step to recruitment of silencing factors to the distant *Slc22a2* and *Slc22a3* genes. Two findings argue against this model; the *Air* promoter is included in the transcription overlap but not silenced, and imprinted expression of *Air*, *Slc22a2* and *Slc22a3* is maintained in the absence of the *Igf2r*/*Air* transcription overlap (Sleutels *et al*, 2003).

The third model of 'RNA-directed targeting' is based on the strong similarities between genomic imprinting and X inactivation (Reik and Lewis, 2005). This model proposes that the *Air* ncRNA is localized to the silenced 300 kb region and attracts repressive chromatin proteins to the three silenced genes. The most prominent example of 'RNA-directed targeting' is mammalian XCI in which the *Xist* ncRNA coats one entire X chromosome and induces gene silencing by recruiting repressive histone modifications and DNA methylation (Heard, 2004). This model has not yet been directly tested for the *Air* ncRNA but analysis of DNase I hypersensitive sites in a 220 kb region that includes the complete *Igf2r*/*Air* genes does not indicate that expression of *Air* recruits silent chromatin to the paternal chromosome (Pauler *et al*, 2005). In addition, the results presented here, on the lack of a correlation between *Air* abundance and the number of genes silenced, combined with the finding that *Air* is unstable, do not indicate a role for the RNA itself.

An earlier suggestion that transcription rather than the RNA induces silencing could not readily be transferred to the whole *Igf2r* cluster, as *Air* does not overlap all silenced genes in the cluster (Rougeulle and Heard, 2002). However, in view of the fact that transcription-based silencing could incorporate instability of the mature unspliced *Air* transcript, we present here a model that is based on 'transcription interference' (TI) (Figure 8). In this model, the *Air* ncRNA is suggested to silence *Igf2r* and the downstream *Slc22a2* and *Slc22a3* genes on the paternal chromosome by the act of transcription through a domain regulatory element. TI has recently received new attention as a regulatory feature in eukaryotic genomes (Shearwin *et al*, 2005) and would depend only on the process of transcription to ensure that RNAPII from the *Air* promoter continually traverses the promoter of *Igf2r*, and a key long-range *cis*-acting domain regulator needed for placental expression of the *Slc22a2* and *Slc22a3* genes (Figure 8). This putative *cis*-acting domain regulator has not yet been identified and is predicted to lie anywhere within the 108 kb *Air* transcription unit. As the nature of this element may not correspond to a known *cis*-regulator such as an enhancer and it likely acts only in the placenta, we are currently pursuing genetic approaches in mice to determine functional regions within *Air* to test for the existence of an independent *cis*-regulatory element. Although the instability of the *Air* ncRNA indicates some support for a TI model, the nuclear retention of the transcript does not. However, as argued above, nuclear retention of *Air* may simply be a consequence of lack of splicing.

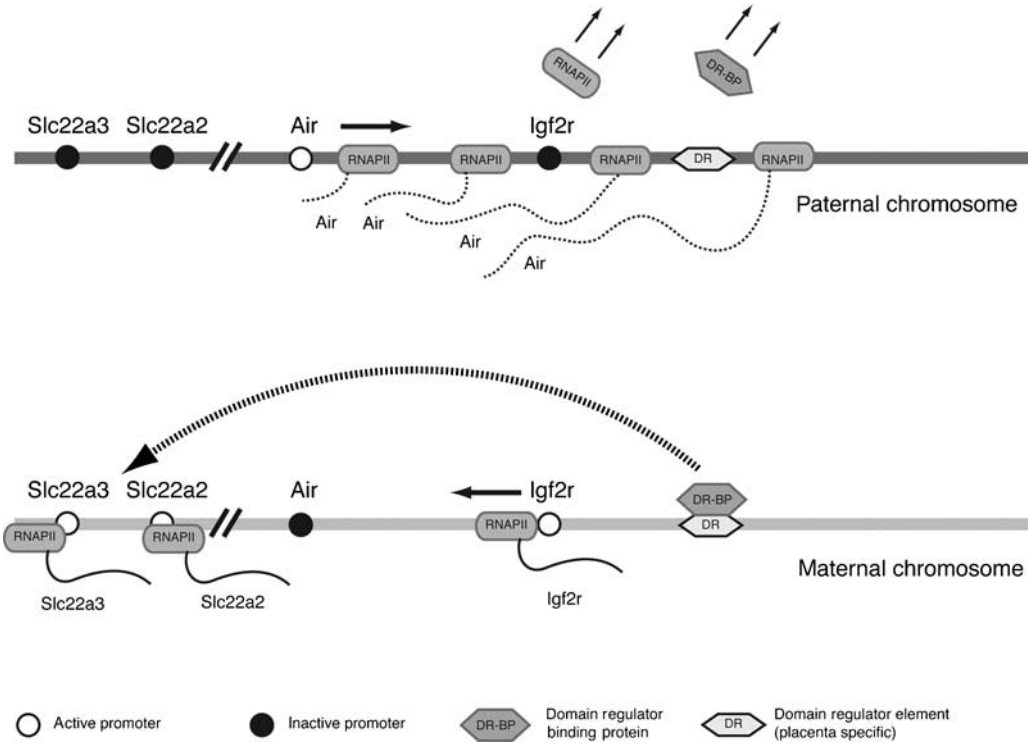


Figure 8 TI model of *Air* ncRNA-mediated gene silencing. The expression pattern of 11.5 dpc placenta is shown (note this is the only embryonic tissue to express *Slc22a2* and *Slc22a3*). On the paternal chromosome, transcription of the *Air* ncRNA (RNAPII with dotted lines) is predicted to interfere with RNAPII binding to the *Igf2r* promoter, and to interfere with activation of a placental-specific domain regulator (DR) needed to express the *Slc22a2* and *Slc22a3* genes. On the maternal chromosome, the *Air* promoter is pre-emptively silenced by a DNA methylation imprint acquired in the oocyte. RNAPII can access the *Igf2r* promoter and domain regulator binding proteins (DR-BP) can access the placental-specific domain regulator and activate the *Slc22a2* and *Slc22a3* genes (striped arrow). A domain regulator is envisaged to be a *cis*-acting element such as an enhancer, or a less well-defined element such as a matrix attachment region or may even be an unknown *cis*-acting element. The low stability of the *Air* ncRNA is indicated by dotted lines of the nascent transcript.

Although *Air* is often compared to the *Xist* ncRNA, the results presented here indicate greater similarities to the *Tsix* ncRNA. These similarities include an antisense overlap with a silenced sense gene, inefficient splicing, a short half-life and nuclear retention. *Tsix* has been shown to function at the level of the RNA by recruiting a DNA methylating enzyme and has also been suggested to induce transient chromatin changes by transcription through the *Xist* promoter (Sun *et al*, 2006). The silencing function of *Tsix*, however, is limited to the overlapped *Xist* gene, whereas *Air* can also silence non-overlapped genes in a methylation-independent manner. Thus, the details of the two systems are likely to differ. In summary, this characterization of the *Air* ncRNA favours a role for *Air* transcription in the silencing mechanism but does not exclude a role for the RNA itself, and it remains possible that *Air* could act at different levels to mediate *cis*-acting silencing of the *Igf2r* cluster. The data presented here provide a base for further studies that will allow features of the TI model to be compared with other models of ncRNA-mediated gene silencing at this and other imprinted gene clusters.

Materials and methods

Cells, RNA and transcription inhibitors

MEFs used: NIH3T3 (+/+), MEFF (+/Thp), Thp/DB104 and DB104/Thp. (+) wild-type chromosome 17; the Thp chromosome contains a 6 Mbp deletion that contains the complete *Igf2r*/*Air* imprinted cluster and DB104 contains a two-copy transgene each carrying a fully imprinted *Igf2r* cluster (Wutz *et al*, 1997). Primary MEFs used for Figure 6 were prepared from seven 13.5 dpc FVB embryos and used at p2. Cytoplasmic and nuclear RNA was prepared using either the *Paris*^{TM} kit (Ambion) or standard protocols. For Actinomycin D and α-amanitin treatment, 5×10^5 cells seeded per 10 cm dish were cultured for 42 h. At time point 0, the media were removed, cells washed with PBS and incubated with media supplemented with 10 μg/ml Actinomycin D or 5 μg/ml α-amanitin (both dissolved in ethanol). Control dishes were incubated with media plus ethanol. At each time point, cells from a control dish and a treated dish were harvested for RNA using standard techniques. RNA blots and RPA were performed using standard techniques (probes are described in Supplementary data).

RNA analysis

RNA blot refers to 'Northern' blot that were prepared from formaldehyde gels in standard conditions. RPA refers to 'RNase protection assay' that was performed using the RPA III^{TM}-kit (Ambion) according to instructions. RPA probe name, accession number, position, and size of the unprotected (upr) and protected (pr) bands are given in Supplementary data. RNA blot and RPA signals were obtained from Phosphoimager screens scanned by a Typhoon8600 scanner (Molecular Dynamics, Amersham) and quantified by ImageJ software (http://rsb.info.nih.gov/ij/index.html).

Real-time qPCR

Taqman probes and primers were designed by PrimerExpress and qPCR performed with the ABI PRISM 7000 with the following primers (900 nM) and Taqman probe (200 nM) under the following cycling conditions: 2 min 50°C, 10 min 95°C, 40 cycles of 15 s 95°C and 1 min 60°C (62°C for *Air* 5′ and splice variants). RNA quantification was made by the standard curve method using serial

dilutions of plasmids (*Air 5′* and splice variants) or cDNA (adult mouse heart or total NIH3T3 RNA for *Air* middle, *Gapdh* ex5, *Igf2r* ex48, *CypA* ex3/4, *45S* pre-rRNA, *18S* rRNA). Assay specificity for full-length unspliced and spliced *Air* variants was tested by serial dilutions of plasmids. Relative quantification and statistics were performed as described in the manufacturer's protocol (Applied Biosystems). Primers and Taqman probes used for qPCR assays are described in Supplementary data.

Immunoprecipitation

IP was performed with mouse monoclonal antibody (clone H20, cat. no. 201001, Synaptic Systems) to 3mG and 7mGcap structures and 10 μg DNase I-treated total RNA from NIH3T3 cells. Equal volumes of the supernatant, first and fifth wash and bound RNA were analysed after reverse transcription by qPCR after precipitation and resuspension in equal volumes. Three biological replicates were performed each including a mock IP control (beads without antibody) and an IP with an unrelated antibody of the same isotype (IgG1, MeCP2, Upstate) processed simultaneously.

EST search and promoter analysis

Transcription factor binding sites at the *Air* ncRNA promoter were determined by MatInspector (www.genomatix.de). EST sequences that align to the sequence between *Mas1* and exon 3 of *Igf2r* found by the Ensembl 30 database (http://www.ensemble.org) were tested for discontinuous alignment to *Air* (AJ249895) by PipMaker (http://pipmaker.bx.psu.edu/pipmaker/). Spliced ESTs were additionally aligned against the Blast database (http://www.ncbi.nlm.nih.gov/BLAST/) to find similar sequences. Repeat content was analysed by Repeat masker (http://www.repeatmasker.org/).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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